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Replication-competent retrovirus vector-mediated prodrug activator gene therapy in experimental models of human malignant mesothelioma

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Abstract

Replication-competent retrovirus (RCR) vectors have been shown to achieve significantly enhanced tumor transduction efficiency and therapeutic efficacy in various cancer models. In the present study, we investigated RCR vector-mediated prodrug activator gene therapy for the treatment of malignant mesothelioma, a highly aggressive tumor with poor prognosis. RCR vector expressing the green fluorescent protein marker gene (RCR-GFP) successfully infected and efficiently replicated in human malignant mesothelioma cell lines, as compared to non-malignant mesothelial cells *in vitro*. In mice with pre-established subcutaneous tumor xenografts, RCR-GFP vector showed robust spread throughout entire tumor masses after intratumoral administration. Next, RCR-CD, expressing the yeast cytosine deaminase (CD) prodrug activator gene, showed efficient transmission of the prodrug activator gene associated with replicative spread of the virus, resulting in efficient killing of malignant mesothelioma cells in a prodrug 5-fluorocytosine (5FC)-dose dependent manner *in vitro*. After a single intratumoral injection of RCR-CD followed by intraperitoneal administration of 5FC, RCR vector-mediated prodrug activator gene therapy achieved significant inhibition of subcutaneous tumor growth, and significantly prolonged survival in the disseminated peritoneal model of malignant mesothelioma. These data indicate the potential utility of RCR vector-mediated prodrug activator gene therapy in the treatment of malignant mesothelioma.

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Conflict of interest

The authors declare no conflict of interest.

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Keywords

replication-competent retrovirus vector; prodrug activator gene therapy; molecular imaging; malignant; mesothelioma

Introduction

Malignant mesothelioma is an aggressive cancer that arises from the mesothelial cells of serous membranes lining the pleural, peritoneal, and pericardial cavities. Pleural mesothelioma is one of the most lethal cancers, and in most cases caused by previous exposure to asbestos [1, 2]. Conventional therapies for this malignancy include surgical resection, chemotherapy, and irradiation, but these measures are generally non-curative [2, 3]. Recent randomized studies with combined chemotherapy using cisplatin and antifolate drugs have demonstrated some survival benefit for patients with mesothelioma [4], but none of these treatments significantly impacts upon the progression and the outcome of mesothelioma. Moreover, this disease is increasing in frequency throughout the world [1, 2], with the incidence of pleural mesothelioma predicted to peak over the next 10–20 years [1, 2, 5]. As a result, new therapeutic paradigms and alternative treatment options are urgently needed for more effective treatment of this aggressive and currently incurable malignancy.

An emerging technology that shows considerable promise as a novel treatment option is use of oncolytic viruses that are capable of tumor-selective replication [6, 7]. This strategy is based on the observation that most tumor cells have impaired antiviral responses that makes them more sensitive for viruses [3]. For experimental gene therapy for mesothelioma, various viruses have been utilized for development of oncolytic viruses as replicating viruses, including adenovirus [8, 9], vaccinia virus [10], herpes simplex virus [11], and vesicular stomatitis virus [12]. Recently, we have demonstrated that murine leukemia virus (MLV)-based RCR vectors show tumor selectivity due to an inherent and stringent specificity for mitotically active cells [13]. In contrast to other viruses used in cancer virotherapy, MLV-based RCRs are non-cytolytic by nature, but can be engineered to carry prodrug activator genes, which mediate synchronized cell killing of infected tumor cells upon prodrug administration. Using RCR vectors expressing the yeast cytosine deaminase (CD) prodrug activator gene, we have demonstrated highly efficient killing of a wide variety of cancer cells both *in vitro* and *in vivo* upon administration of its prodrug, 5-fluorocytosine (5FC) [14–17]. Furthermore, as RCR vectors stably integrate into the host cell genome, residual infected cancer cells serve as a reservoir for long-term viral persistence and further viral spread, even to metastatic sites. For example, after a single stereotactic intratumoral injection of RCR vector delivering the CD prodrug activator gene into orthotopically xenografted human gliomas, viral spread was observed in the majority of secondary tumor foci that had migrated away from the primary tumor site, thereby enabling multiple cycles of 5FC prodrug administration at periodic intervals to achieve 100 % survival for >100 days, as compared to 0 % survival of control groups in < 40 days [16]. Based on these promising preclinical results, a clinical trial for RCR vector-mediated prodrug activator gene therapy in patients with recurrent glioblastoma has recently been approved by the FDA.

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In the present study, we tested the replication kinetics, transduction efficiency, and tumor-selectivity of RCR vector in human malignant mesothelioma cells in culture and *in vivo*. We also evaluated tumor growth inhibition and prolonged survival in a peritoneally-disseminated multiple tumor model of human malignant mesothelioma. This represents the first study showing the efficient transduction of mesothelioma cells by RCR vectors and the therapeutic efficacy of RCR vector-mediated CD/5FC prodrug activator gene therapy in both local subcutaneous and abdominally disseminated experimental models of human mesothelioma.

Materials and Methods

Cell lines

Normal human adult mesothelial cells (NMC) were purchased from Zen-Bio, Inc. (Research Triangle Park, NC, USA) and maintained in Mesothelial Cell Growth Medium (Zen-Bio, Inc.). Non-malignant human pleural mesothelial cells transformed with SV40 T antigen (Met5A) and three human mesothelioma cell lines, MSTO-211H, NCI-H2052 (H2052), and NCI-H2452 (H2452) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were grown in RPMI 1640 (Nacalai Tesque, Kyoto, Japan) supplemented with 10 % fetal calf serum (FCS; HyClone, Logan, UT, USA). Transformed human embryonic kidney 293T cell lines were cultured in Dulbecco's modified Eagle's medium (Nacalai Tesque) supplemented with 10 % FCS. All the cells were grown in 5 % CO_2 at 37°C.

Viral vector plasmid and virus production

The RCR vector plasmids, pRCR-GFP and pRCR-CD, have been described previously as ACE-emd and ACE-CD, respectively [17, 18]; each contains a full-length replication-competent amphotropic MLV provirus with an additional internal ribosome entry site (IRES)- green fluorescent protein marker gene (GFP) or IRES-CD cassette, respectively (Figures. 1a and 3a). For virus production, 293T cells were transiently transfected with pRCR-GFP or pRCR-CD using LipofectAMINE 2000 (Life Technologies Japan, Tokyo, Japan), replenished with serum-free medium, and 48 h later the supernatant medium was harvested, filtered, and stored frozen at −80°C. These vectors were prepared side by side and confirmed to have comparable titers by quantitative real-time PCR (data not shown).

A self-inactivating lentivirus vector expressing the fluorescent protein mCherry, LVSKc-mCherry, was created from sinSKcmv-EGFP (LV-GFP) as described previously [19], by replacement of GFP with mCherry cDNA. The virus preparations were produced by transient cotransfection of 293T cells as described previously [14, 19, 20]. The titers of these vectors were determined by fluorescent protein expression using a FACScalibur flow cytometer (Becton Dickinson Japan, Tokyo, Japan) and expressed as transducing units (TU) per mL.

Replication kinetics of RCR *in vitro*

For analysis of replication kinetics *in vitro*, virus vector stock at an MOI of 0.01 was used for infection of various human cell lines at 20 % confluency. At serial time points, the cells

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were trypsinized, one-fourth of the cells were replated, and the remainder analyzed for GFP expression by flow cytometry as above.

Replication kinetics in subcutaneous tumor models

BALB/c-nu/nu (nude) mice (Charles River Japan Co., Yokohama, Japan) were bred and maintained under specific pathogen-free conditions, and all studies conducted under protocols approved by the Hyogo College of Medicine Animal Research Committee. Human malignant mesothelioma MSTO-211H xenografts were established in 6- to 8-week-old female nude mice by subcutaneous inoculation of 1×10^6 MSTO-211H cells into the right dorsal flank. When tumors reached a diameter of ~5 mm, three groups of mice (n=3 per group) mice were injected intratumorally with 50 μL of PBS, LV-GFP (1×10^4 TU) or RCR-GFP (1×10^4 TU) on Day 0. Over sequential days, mice were anesthetized with sodium pentobarbital, and spectral fluorescence imaging was performed using the Maestro *in vivo* fluorescence imaging system (Cambridge Research and Instrumentation, Woburn, MA, USA), as previously described [21, 22]. For detection of GFP fluorescence (maximum excitation 488 nm; maximum emission 507 nm), whole body images (0.05- to 0.5-second exposure) were captured at a 500–600 nm range in 10 nm steps with a band-pass filter from 445–490 nm and a long-pass filter over 515 nm). All images were analyzed by Maestro 2.2 software in order to create spectral unmixed images of fluorescein and autofluorescence. To evaluate the increase of fluorescence intensities in tumors, the total signal intensities (scaled counts/sec) were divided by tumor volumes (mm^3). Tumors were harvested on Day 19, and immediately digested with collagenase into cell suspensions for analysis of GFP expression by flow cytometry.

Cytotoxicity assay *in vitro*

For quantitative analysis of the drug cytotoxicity, triplicate wells containing human MSTO-211H, H2052, H2452 or Met5A cells (1×10^4 cells/well) which had been pretransduced with RCR-CD at an MOI of 0.01 and maintained for 15 days, were cultured in 96-well tissue-culture plates with various concentrations of 5FC. On Day 3, the viable cell numbers of triplicate cultures were measured by the Alamar Blue method according to the manufacturer's instructions (Alamar Biosciences, Inc., Sacramento, CA). Briefly, 40 μL of Alamar Blue was aseptically added to the cultures, which were then returned to the incubator for 3 h; fluorescence was measured by an ARVO X4 multilabel plate reader with a 544 nm excitation wavelength and a 590 nm emission wavelength (PerkinElmer Japan, Tokyo, Japan). The percentage of viable cells was determined by calculation of the fluorescence of viable cells as measured against wells containing no 5FC.

Prodrug activator gene therapy in subcutaneous and intraperitoneal tumor models

MSTO-211H cells were grown subcutaneously in nude mice to a diameter of 5–6 mm as above, and three groups of mice (n=5 per group) were then injected intratumorally with 50 μL of PBS, RCR-GFP (1×10^4 TU) or RCR-CD on Day 0, followed by intraperitoneal administration of either 5FC (500 mg/kg/day) from Day 12 to Day 32. The mice were observed closely and the tumors were measured twice a week. The tumor volume was

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calculated as $a \times b^2 \times 0.5$, where *a* and *b* were the largest and smallest diameters, respectively.

For intraperitoneal tumor models, we first produced a stable cell population of MSTO-211H which express mCherry (MSTO-mCherry) by transduction with LVSKc-mCherry. MSTO-mCherry cells were then infected by RCR-GFP (n = 12) or RCR-CD (n = 9), and maintained for 4 weeks to create stably transduced cell populations, MSTO-mCherry/GFP or MSTO-mCherry/CD, respectively. The MSTO-mCherry/GFP or MSTO-mCherry/CD cells were mixed with uninfected parental MSTO-mCherry cells at a ratio of 1 : 99 and these MSTO-211H cell mixtures (5×10^4 cells) in 500 μL PBS were intraperitoneally injected into nude mice. Two weeks later, tumor formation was confirmed in all mice by *in vivo* imaging, and daily intraperitoneal 5FC administration was initiated (500 mg/kg/day). Over sequential weeks, spectral fluorescence imaging was performed to detect tumors positive for mCherry (maximum excitation 587 nm; maximum emission 610 nm). Whole body images (0.05- to 0.5-second exposure) were captured at a 550–800 nm range in 10 nm steps with a band-pass filter from 503–555 nm and a long-pass filter over 580 nm.

The following criteria were established to determine when an animal's health was so poor that it needed to be euthanized. When the animals demonstrated, in order, paleness of the paws and muzzle, weight loss. lethargy/cachexia, and finally hypothermia, they were necropsied.

Statistical analysis

The results are presented as mean ± SD. Statistical significance of differences was calculated using Student's *t*-test or one-way ANOVA, and a *P* value of <0.01 was considered significant in all analyses, which were done with Prism 4 statistical software (GraphPad Software).

Results

RCR vectors replicate efficiently in human malignant mesothelioma cell lines

To evaluate replication kinetics in human malignant mesothelioma cells, we first used the MLV-based RCR vector RCR-GFP, which contains an IRES-GFP cassette inserted precisely at the env-3'untranslated region (UTR) boundary (Figure 1a). GFP expression was monitored by flow cytometry at serial time points after inoculation of cells with RCR-GFP at an MOI of 0.01 (Figure 1b). In MSTO-211H, H2052, and H2452 human malignant mesothelioma cells, the percentage of GFP-expressing cells quickly increased in a logarithmic manner and reached > 80 % within 15 days and > 90 % within 18 days after virus inoculation, and then remained stable thereafter. By contrast, in normal adult mesothelial cells (NMC) and non-malignant transformed pleural mesothelial cells (Met5A), there is a prolonged lag time before logarithmic spread of the virus, resulting in a large discrepancy of RCR spread between the malignant mesothelioma cell lines and the non-malignant cells. Thus, the RCR vector was capable of efficient replication and progressive spread through malignant mesothelioma cells *in vitro*.

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To examine the spread of RCR vectors *in vivo*, nude mice with subcutaneous MSTO-211H tumors received a single intratumoral injection of RCR-GFP, LV-GFP replication-defective lentiviral vector, or PBS vehicle control on Day 0, and GFP expression was monitored at serial time points thereafter by *in vivo* fluorescence imaging in a non-invasive manner (Figure 2a). In mice injected with the replication-defective lentiviral vector, LV-GFP, intratumoral GFP expression was observed on Day 3, and proportionally increased with an increase in tumor size. In contrast, in all animals injected with RCR vector, intratumoral GFP expression increased robustly and reached throughout entire tumor by Day 12, without detectable signal in extratumoral normal tissue. The fluorescence intensities were normalized to the tumor volumes and found to be highly increased in RCR-GFP-injected tumors compared to LV-GFP-injected tumors (Figure 2b), indicating efficient RCR spread in these tumors. On Day 19, tumors were harvested, immediately digested with collagenase into cell suspensions, and the percentage of GFP-positive cells was 5.5 ± 2.9 % with LV-GFP vs. 95.8 ± 6.5 % with RCR-GFP by flow cytometry.

These results were consistent with the results *in vitro* RCR replication kinetics in MSTO-211H, and indicate that the RCR vector was also capable of efficient replication and progressive spread through mesothelioma tumors *in vivo*. Thus, the RCR vector could achieve robust spread both *in vitro* and *in vivo*, as confirmed by GFP transmission.

Prodrug activator gene-mediated cell killing effect of RCR in human malignant mesothelioma cells

To investigate the efficacy of RCR-mediated prodrug activator gene therapy in human malignant mesothelioma cells, we used RCR-CD, which expresses the CD prodrug activator gene (Figure 3a). The human mesothelioma cells were infected with RCR-CD at an MOI of 0.01 on Day 0 and exposed to the 5FC prodrug at various concentrations from Day 15 for three days. On Day 18, cell viability was examined by Alamar blue assay. In malignant mesothelioma cell lines (MSTO-211H, H2052, and H2452), decreased cell viability was observed in a 5FC-dose-dependent manner (Figure 3b). At the highest 5FC dose level (10mM), the viability of all RCR-CD-transduced malignant mesothelioma cells was significantly decreased to < 30 %, while RCR-CD-transduced non-malignant Met5A cells showed significantly higher levels of survival (~80 %). These results were consistent with *in vitro* RCR-GFP replication kinetics in these cells (Fig. 1b), and indicate that the RCR-CD vector could achieve selective spread and cytotoxicity in malignant mesothelioma cells.

RCR-mediated CD/5FC prodrug activator gene therapy shows potent *in vivo* antitumor effects in subcutaneous human mesothelioma xenograft models

To examine the antitumoral therapeutic efficacy of RCR-mediated CD/5FC prodrug activator gene therapy, nude mice bearing established MSTO-211H tumors were treated with a single intratumoral injection (1×10^4 TU total dose) of either RCR-GFP or RCR-CD, or of PBS vehicle control on Day 0, followed by intraperitoneal administration of 5FC. As shown in Figure 4, subcutaneous tumors treated with RCR-GFP showed no obvious inhibition of tumor growth after 5FC administration, as compared with the PBS control group. In contrast, the growth of RCR-CD transduced tumors was significantly inhibited by 5FC treatment ($P<0.01$ after Day 21). Thus, RCR-mediated prodrug activator gene therapy

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could achieve effective *in vivo* growth inhibition in this subcutaneous tumor model of human malignant mesothelioma.

Extended survival in human intraperitoneal mesohelioma xenograft models by RCR-mediated CD/5FC prodrug activator gene therapy

We next assessed the therapeutic effect of RCR vector-mediated prodrug activator gene therapy in a peritoneally disseminated mesothelioma model, using MSTO-211H cells pretransduced with a conventional replication-defective lentivirus vector expressing mCherry fluorescent protein (MSTO-mCherry). Stable expression of mCherry in these cells was confirmed by flow cytometry (data not shown), and allows monitoring of individual mice over serial time points by *in vivo* fluorescence imaging.

MSTO-mCherry cells were fully transduced by RCR-GFP or RCR-CD, creating MSTO-mCherry/GFP or MSTO-mCherry/CD cells, respectively. These RCR-transduced MSTO-mCherry cells and parent MSTO-mCherry cells were mixed at a low percentage of transduction (~1 %), and this cell mixture was intraperitoneally injected in nude mice. Fourteen days later, tumor formation was confirmed by *in vivo* imaging, and intraperitoneal 5FC administration was initiated (500 mg/kg/day) for 15 consecutive days. In control mice bearing MSTO-mCherry/GFP tumors with 5FC, both progressive tumor growth and robust spread of the non-therapeutic RCR-GFP vector in the tumor was observed by *in vivo* imaging, although only the biggest tumor can be visible at short exposure time as shown in Figure 5a. On Day 61, after death of the last survivor in the RCR-GFP group, eight tumors were harvested and immediately digested with collagenase into cell suspensions. These tumors all showed high levels of GFP expression, averaging 84.8 ± 12.5 % by flow cytometry. By contrast, in four out of nine mice bearing MSTO-mCherry/CD tumors which survived until sacrifice on Day 100 with 5FC administration, tumors were initially visible by *in vivo* fluorescence imaging, but showed subsequent regression, and by Day 100, no visible tumors were observed. Furthermore, the MSTO-mCherry/CD group showed significantly prolonged median survival compared to the MSTO-mCherry/GFP group (MSTO-mCherry/CD: 81 days vs. MSTO-mCherry/GFP: 34.5 days, $P<0.01$; Figure 5b). Thus, after 5FC prodrug administration, RCR-CD achieved significant tumor growth inhibition and prolonged survival after implantation of human malignant mesothelioma cells pretransduced with virus at doses as low as 1 %.

Discussion

Malignant mesothelioma is an attractive target for gene therapy based on the following: (1) it is an aggressive neoplasm with poor prognosis (median survival of 1–2 years after initial diagnosis), (2) the tumor is usually localized in a cavity, and vectors can be easily administered simply via a drainage tube, which also makes it easy to monitor the status of the lesion treated [3, 23], and (3) morbidity and mortality are primarily related to regional tumor extension [24]. Several gene therapy strategies for mesothelioma are being tested in clinical trials, and have experienced some success. The first clinical trial involved prodrug activator gene therapy mediated by a replication-defective adenoviral vector expressing the herpes simplex virus thymidine kinase gene (HSV-TK) [24, 25]. Through a series of Phase I

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clinical trials, among a total of 34 patients receiving the adenoviral vector-mediated HSV-TK prodrug activator gene therapy, four achieved considerable tumor regression, and two patients achieved complete tumor regression for more than seven years after treatment [24]. Dose-related intratumoral HSV-TK gene transfer was demonstrated in most patients who received intrathoracic administration with the vector, but HSV-TK protein expression proved relatively superficial at tumor surfaces up to 20–50 cell layers deep by immunohistochemical assessment [25]. Hence in this case, immunological mechanisms are likely to have accounted for any therapeutic effect achieved, and subsequent trials have focused on adenoviral delivery of immunostimulatory genes [26, 27].

For the ultimate success of prodrug activator gene therapy for cancer, improved overall transduction efficiency throughout the entire tumor is required. However, human clinical trials have documented that even high-titer adenoviral vectors only achieve limited transduction into tumor cells and this is confined to areas surrounding the needle track. It may therefore be technically difficult to achieve efficacious levels of gene delivery by conventional replication-defective vectors, because virus diffusion in the tumor is limited and pleural mesothelioma is usually widely spread over a large area.

We found that the human malignant mesothelioma cells tested in this study are highly permissive for RCR, as compared to non-malignant SV40 T antigen-transformed Met5A cells and primary NMC cells induced to divide in culture (Figure 1b). RCR has the capability to permanently integrate into the genome of the host tumor cell and will continue to produce progeny virus transcribed from the integrated copy whether the infected tumor cell itself continues to divide or not. Thus, once any tumor cells have been infected, these will serve as a stable source for continued virus production so that the virus will still be available when adjacent uninfected tumor cells eventually divide, even if this occurs slowly. Indeed, following quite low levels of initial RCR transduction, RCR are shown to be able to spread efficiently *in vivo* in human malignant mesothelioma xenografts (> 95 % transduction efficiency, Figure 2) after virus injection at doses as low as 1×10^4 TU, and in abdominal disseminated tumors (> 80 % transduction efficiency, Figure 5) with initial transduction levels as low as 1 %. When bystander effects are also taken into consideration, such high levels of tumor transduction may achieve sufficient tumor cell killing to destroy dormant cancer stem cells by prodrug activator gene therapy.

In order to evaluate the therapeutic efficacy of RCR-CD vector, we used RCR vector to pretransduce MSTO-mCherry tumor cells. This is based on the highly aggressive nature of MSTO-211H tumor growth; multifocal tumor growth in the peritoneal cavity resulted in lethality within 9 weeks after tumor cell inoculation (median survival of 34.5-day in MSTO-mCherry/GFP group; Figure 5b). Hiraoka et al. have previously reported hepatic metastasis models of murine CT26 colorectal cancer in which the transduction level among different tumor nodules was quite variable after intrasplenic injection of RCR vector [28]. This is partly because of the highly aggressive nature of tumor growth of CT26 cancer cells, therefore the virus could spread for only a limited period of time before the tumor burden became lethal [28]. In our abdominally disseminated mesothelioma models, we used RCR vector-pretransduced MSTO-mCherry tumor cells to evaluate the therapeutic efficacy of RCR-CD prodrug activator gene therapy, because the parent MSTO-211H cells were shown to be

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permissive for vector spread, but also grow as aggressively as CT26 cells. In clinical situations, however, tumor growth is generally slow in the majority of patients with malignant mesothelioma, and this is predicted to affect the replication kinetics of RCR spread within the tumor mass. Although RCR spread will be slower, once infected, the integrated provirus will continue to be expressed continuously from the mesothelioma cells, and the slower rate of tumor growth also allows a longer time period for intratumoral spread after initial viral injection, which should enable transduction of larger numbers of cancer cells even in initially poorly transduced tumor nodules. In a clinical setting, an endoscopic injection of RCR vector into some large tumor nodules in disseminated cases would be beneficial, even if complete responses are not achieved, since the use of RCR vector–mediated prodrug activator gene therapy as a relatively nontoxic neoadjuvant could reduce tumor burden sufficiently to convert an unresectable case to one that may be amenable to subsequent surgical removal.

In conclusion, our results show that RCR vectors can efficiently replicate and achieve significant levels of tumor transduction in human malignant mesothelioma cells, with minimal spread in primary normal human mesothelial epithelial cells, and without detectable spread to normal cells *in vivo*. The present study represents the first study to show efficient transduction of human malignant mesothelioma cells by RCR vectors and the therapeutic efficacy of RCR vector-mediated CD/5FC prodrug activator gene therapy in subcutaneous as well as peritoneally disseminated mesothelioma models *in vivo*. To our knowledge, this study also represents the first report to use two color fluorescence live imaging to monitor tumor growth and virus spread of replicating vector using a disseminated cancer model in abdominal cavity. Our *in vivo* fluorescence imaging mesothelioma models are sensitive and useful tools to monitor tumor growth and to evaluate therapy response in exploring new treatment paradigms for malignant mesothelioma.

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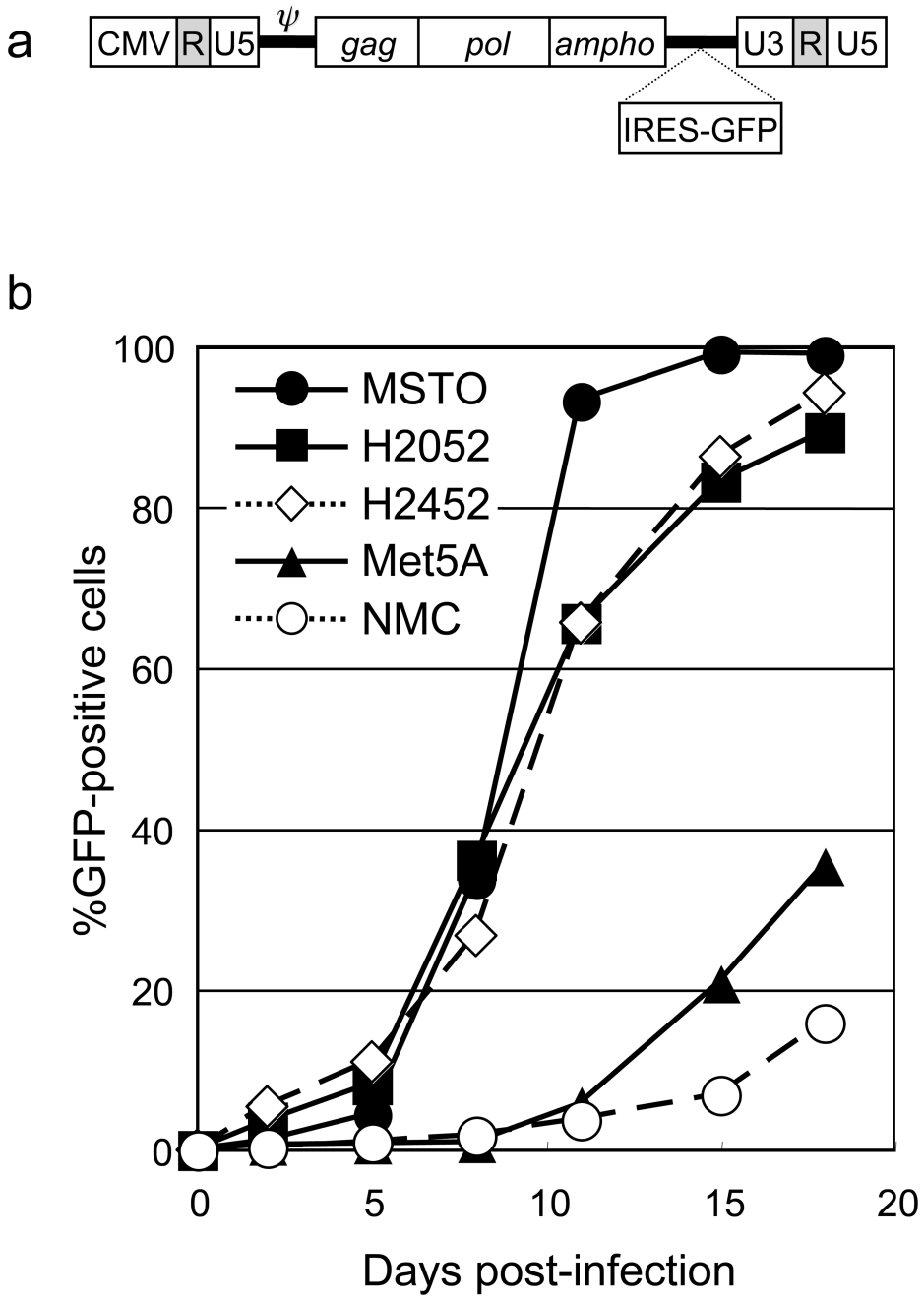

Figure 1. Replication kinetics of RCR vectors in human mesothelioma cells

(a) Schematic structure of RCR-GFP vector. This vector contains a full-length replication-competent amphotropic MLV proviral sequence, in which an IRES-GFP cassette has been inserted between the env gene and 3'-untranslated region, and the U3 region of the 5' long terminal repeat has been replaced by the cytomegalovirus promoter (CMV). ψ: packaging signal, gag-pol: amphotropic MLV genes, IRES: internal ribosome entry site, GFP: green fluorescent protein.

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(b) Replication kinetics of RCR vectors in human mesothelioma cells. Human mesothelioma cells (MSTO, H2052, andH2452) and non-malignant mesothelial cells (Met5A and NMC) were inoculated with RCR-GFP vector at an MOI of 0.01. On the days of passage, cells were analyzed for GFP expression by flow cytometry. Data are representative of 3 independent experiments, all yielding similar results.

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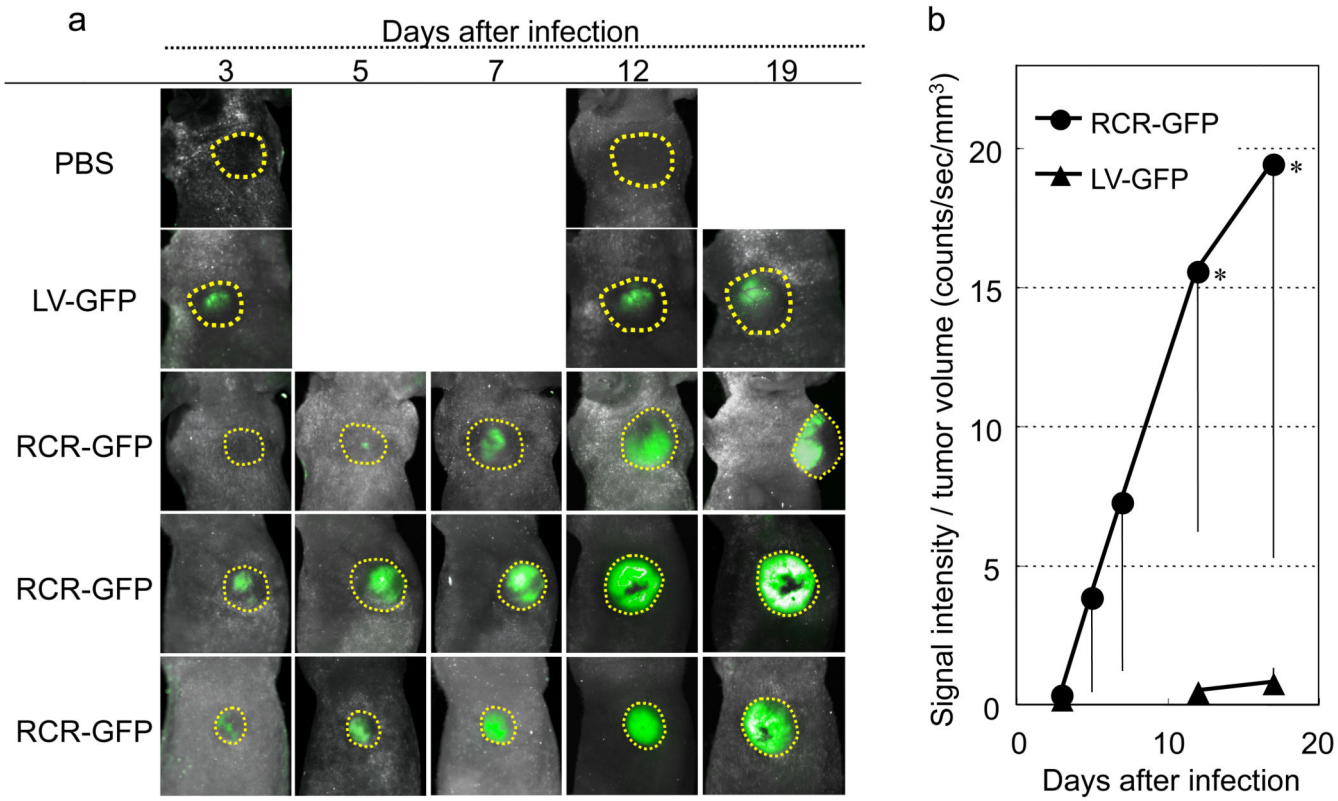

Figure 2. ***In vivo*** **spread of RCR vectors in malignant mesothelioma xenograft tumors**

(a) in vivo fluorescence imaging. Human malignant mesothelioma MSTO-211H tumors were grown subcutaneously in nude mice to 5–6 mm in diameter, and injected intratumorally with 1×10^4 TU (50 μL) of LV-GFP (lentiviral vector), RCR-GFP, or PBS vehicle control on Day 0 (n=3 per group). At different time points indicated in the figure, whole body images (0.05- to 0.5-second exposure) were taken and analyzed by *in vivo* fluorescence imaging system. Representative images are shown from each group. Tumor areas are marked with dotted lines.

(b) Comparison of the fluorescence intensities in tumors injected with LV-GFP and RCR-GFP. The fluorescence intensities were normalized to the tumor volumes. Data shown are averages – (RCR-GFP) or +(LV-GFP) SD from experiments (n=3 per group). *, P <0.01 versus LV-GFP-injected group.

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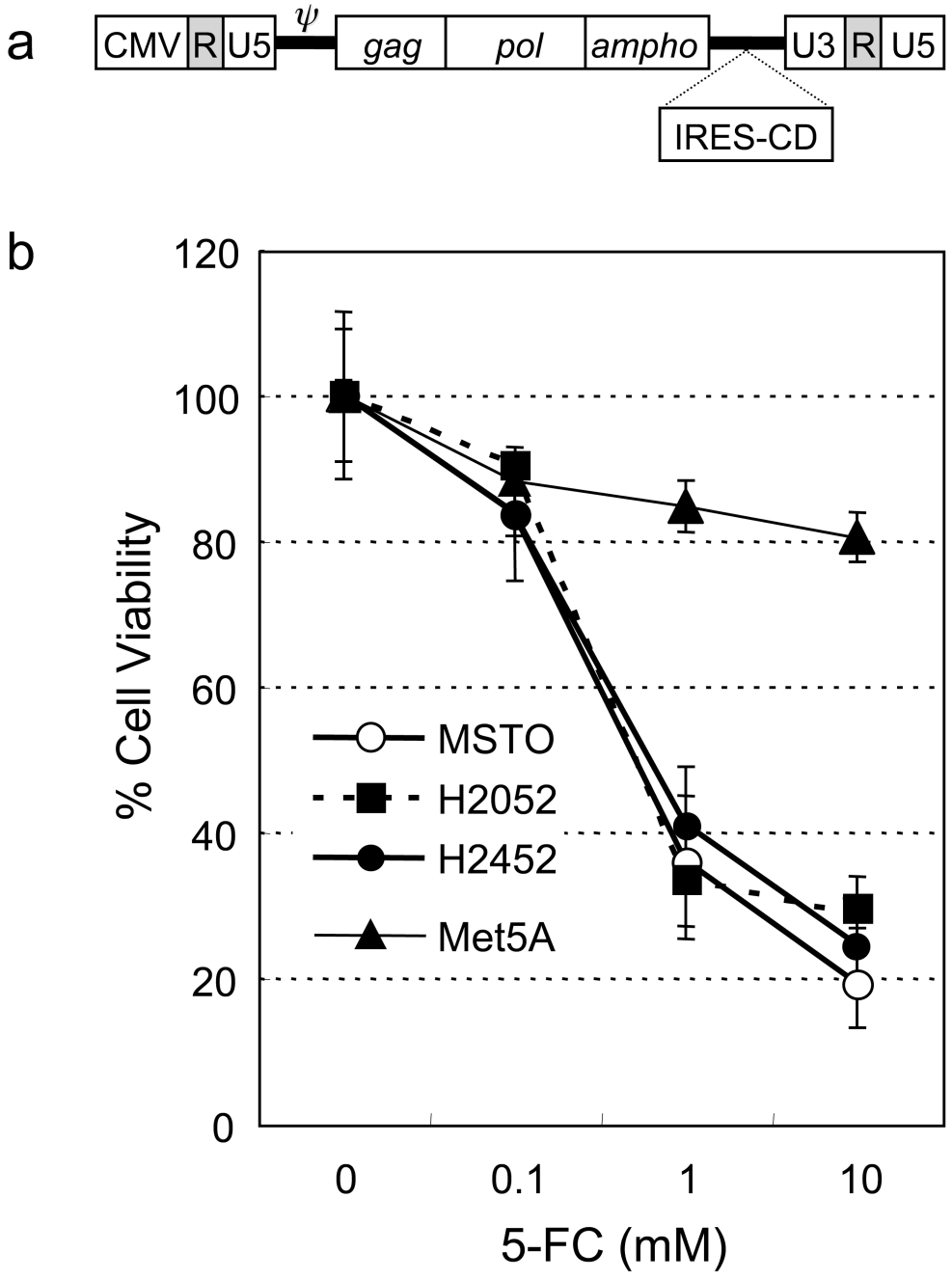

Figure 3. Prodrug activator gene-mediated cell killing effect after RCR infection *in vitro*

(a) Schematic structure of RCR-CD vector. This vector was created by replacement of the IRES-GFP cassette of RCR-GFP with IRES-CD. CD: yeast cytosine deaminase prodrug activator gene.

(b) Cell viability of human mesothelioma cells on day 18 after RCR-CD infection at an MOI of 0.01. Data shown are averages ± SD from experiments performed in triplicate.

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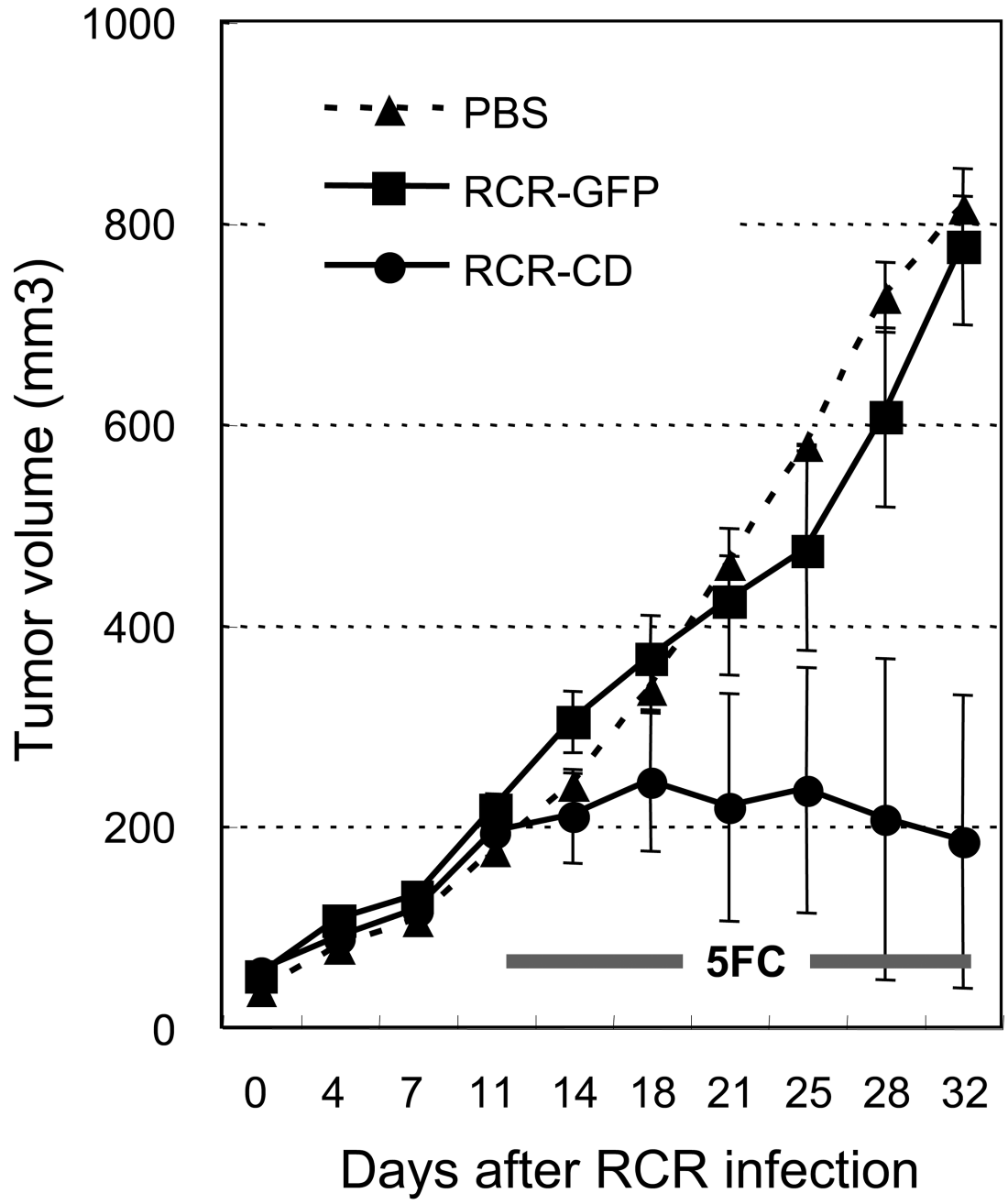

Figure 4. ***In vivo*** **antitumor effect of RCR-mediated prodrug activator gene therapy in subcutaneous xenograft model of human malignant mesothelioma**

MSTO-211H tumors were grown subcutaneously in nude mice to 5–6 mm in diameter, and injected intratumorally with 1×10^4 TU (50 μL) of either RCR-GFP or RCR-CD, or PBS vehicle control on Day 0, followed by intraperitoneal administration of 5FC (500 mg/kg/day) from Day 12 to Day 32 (n=3 per group). Tumor volumes were measured twice a week, and data shown are the mean ± SD.

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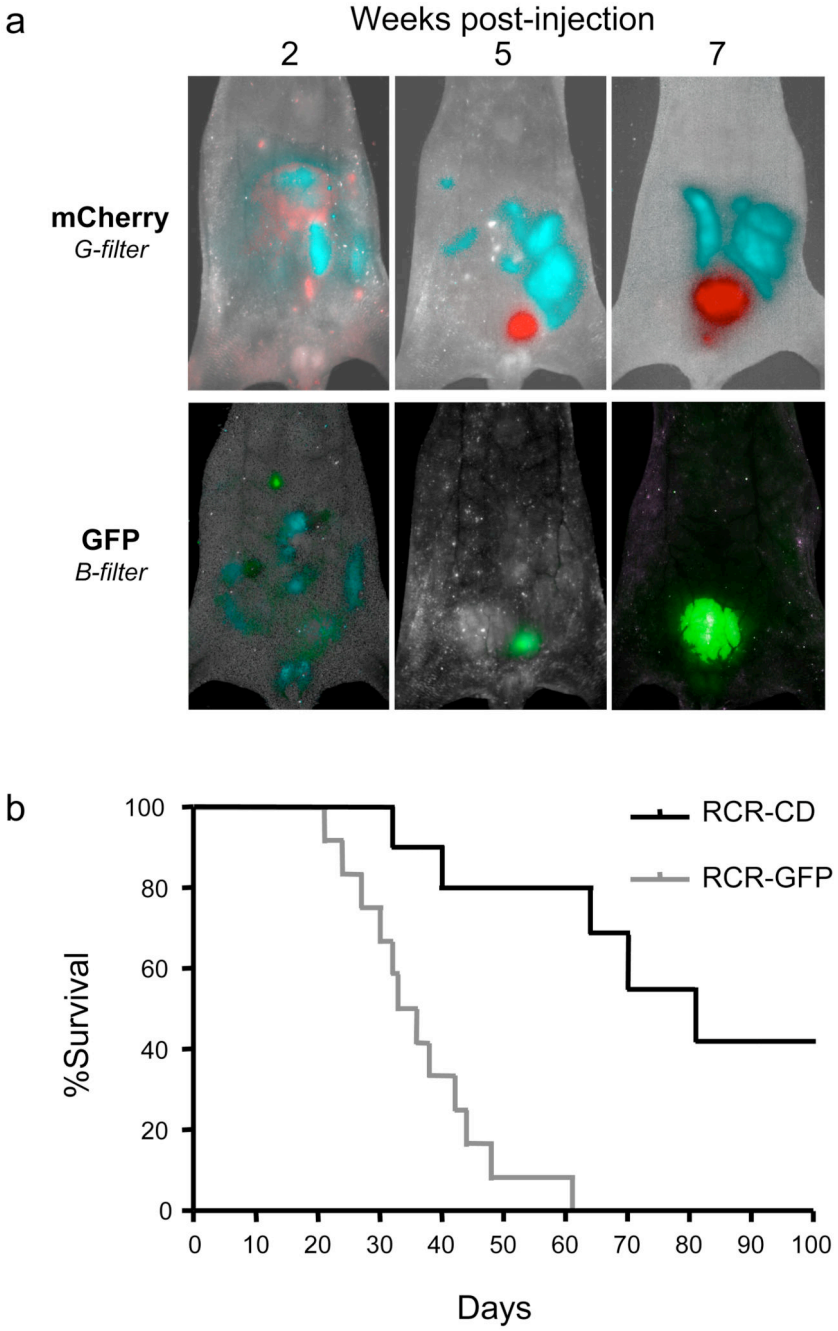

Figure 5. Therapeutic effect of RCR-mediated prodrug activator gene therapy in intraperitoneal xenograft model of human malignant mesothelioma

(a) *In vivo* spread of RCR vectors in intraperitoneal mesothelioma xenograft tumors. Parental MSTO-mCherry cells (99 %) were mixed with MSTO-mCherry infected with RCR-GFP cells (1 %). These cell mixtures were injected intraperitoneously into each mouse on Day 0. Red: mCherry-positive tumors. Green: GFP-positive signal from cells transduced with RCR-GFP. Light blue: background signal from fecal masses.

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(b) Survival after intraperitoneal inoculation of MSTO cells pretransduced with RCR-CD. MSTO-mCherry cells (99 %) were mixed with MSTO-mCherry infected with RCR-GFP or RCR-CD cells (1 %). These cell mixtures were injected intraperitoneally into each mouse (RCR-GFP; n=12, RCR-CD; n=9) on Day 0. Treatment with 5FC prodrug (500 mg/kg/day) was started from Day 14.